EXHIBIT K

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Risk Assessment of N-nitrosodimethylamine Formed Endogenously after Fish-with-Vegetable Meals

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The consumption of fish and nitrate-rich vegetables may lead to the formation of the genotoxic carcinogen N-nitrosodimethylamine (NDMA) in the stomach. To assess human cancer risk associated with this formation, a dynamic *in vitro* gastrointestinal model was used to simulate NDMA formation in the stomach after a fish + vegetable meal. The experimental results were combined with statistical modeling of Dutch food consumption data resulting in predicted exposures to endogenously formed NDMA in the population. The 95th percentile of the long-term exposure distribution was around 4 ng/kg-bw in young children and 0.4 ng/kg-bw in adults. By comparing this exposure with the Benchmark Dose Lower bound (BMDL) 10 for liver cancer in a chronic carcinogenicity study, a chronic margin of exposure (MOE) was calculated of 7000 and 73,000 for young children and adults. Furthermore, the long-term exposure distribution was combined with a dose-response analysis of the liver cancer incidence data to obtain a cancer risk distribution for the human population. The 95th percentile of that distribution was 6×10^{-6} extra risk for 5-year-old children and 8×10^{-7} for adults. The liver cancer data allowed for the analysis of the relationship between tumor incidence and time to tumor. For an extra risk of 10^{-6}, the decrease in time to tumor was conservatively estimated at 3.8 min in the rat, equivalent to 0.1 days in humans. We also combined acute exposure estimates with the BMDL10 from an acute carcinogenicity study for NDMA, resulting in an acute MOE of 110,000. We conclude that the combined consumption of fish and nitrate-rich vegetables appears to lead to marginal increases of additional cancer risk.

Key Words: **N-nitrosodimethylamine; Benchmark dose; probabilistic exposure assessment; risk distribution; time to tumor; MOE; endogenous formation.**

N-nitrosodimethylamine (NDMA) is a potent chronic and acute animal carcinogen (Driver *et al.*, 1987; Peto *et al.*, 1991a,b). NDMA is also classified as a probable human carcinogen (IARC, 1978, 2000). In the human stomach, NDMA may be formed after the consumption of fish and nitrate-rich vegetables (Vermeer, 2000). Nitrate is rapidly taken up into the blood stream and excreted into the saliva where it is transformed by bacteria into nitrite. After swallowing saliva, nitrite reaches the stomach where acid conditions favor its reaction with substances from fish (dimethylamine) to form NDMA. After entering the intestine, a fast neutralization of the pH of the food pulp takes place and the formation of NDMA stops (Mirvish, 1975).

Endogenous formation of NDMA may amount to 27–34 μg (Krul *et al.*, 2004), whereas the direct, exogenous, intake of NDMA in the Netherlands is estimated to be much lower, i.e., around 0.1 μg/day (Ellen *et al.*, 1990). So, although the endogenous NDMA exposure resulting from consumption of fish-vegetable meals is likely to be infrequent, its level is relatively high.

A quantitative cancer risk assessment of endogenously formed NDMA starts with the quantification of NDMA formation related to the consumption of meals of fish with nitrate-rich vegetables. Technical and ethical reasons hamper the direct measurement of NDMA in the stomach after a fish-vegetable meal. Furthermore, the urinary excretion of NDMA only has semiquantitative value here, i.e., it cannot be used for quantitatively estimating the amount of endogenously formed NDMA (Vermeer, 2000). Therefore, other methods need to be considered for quantifying endogenous NDMA formation.

This paper presents a comprehensive risk assessment for endogenously formed NDMA, including an indirect methodology to quantify the exposure, i.e., the amount of NMDA that may be formed in the stomach. We combine the following techniques and approaches. The conversion of nitrate absorbed from food into nitrite in the saliva was estimated using a mathematical biokinetic model that was validated by various studies in human volunteers. The formation of NDMA was measured in an *in vitro* model system under conditions which mimic *in vivo* nitrosation in the human stomach after a fish + vegetable meal as closely as possible. The results were combined with food consumption survey data in the Dutch population to obtain acute and chronic estimates of endogenous NDMA

exposure. The calculated raw intake data were analyzed by a probabilistic exposure model resulting in distributions of long-term average exposures in the population, as well as for acute exposure distributions. Two carcinogenicity studies with NDMA in rats, one after chronic and one after a single acute exposure, were reanalyzed using the Benchmark Dose (BMD) approach. And finally, various risk characterization approaches were applied to evaluate to what extent the Dutch population might be subject to cancer risks because of fish + vegetable meals.

EXPOSURE ASSESSMENT

NDMA Formation from a Fish + Vegetable Meal

We used a dynamic *in vitro* gastrointestinal system developed by Minekus *et al.* (1995) and Minekus (1998) for experimentally estimating the rate of endogenous NDMA formation as a function of the amount of fish consumed and the amount of nitrate ingested. This system mimics the *in vivo* nitrosation better than static *in vitro* models, which lack the peristaltic movement of the food matrix and the dynamics of the stomach's acidity after a meal (Krul *et al.*, 2004). The system consists of the four gastrointestinal compartments: stomach, duodenum, jejunum, and ileum.

A complete description of the experimental procedures involved is presented in Supplementary material A1, A2, and A3. In short, various amounts (30–100 g) of fish (cod, herring, pollack, plaice, mackerel, salmon, pike, tuna, shrimps, or fish fingers) together with food components mimicking a representative adult/child meal were added to the gastric compartment of this system. A solution of nitrite was then gradually infused for a time period of up to 3 h, to mimic the swallowing of nitrite-containing saliva after the meal. After this time period, 95% of the gastric compartment has been emptied into the duodenum compartment of the system, where the formation of NDMA is stopped because of pH neutralization. This material was collected from the duodenum compartment, and the amount of NDMA was measured. The *in vitro* nitrite infusion was varied between equivalents of 0.1 and 10 times the acceptable daily intake (ADI) of nitrate. The tuning of the *in vitro* nitrite infusion to the *in vivo* transport of nitrite with saliva resulting from nitrate intake was based on a toxicokinetic model for nitrate and nitrite in humans (see Supplementary material A2 and A3).

The experimental results were used for deriving a generic statistical relationship describing the amount of NDMA as a function of the amount of fish added to the system and of the simulated nitrate exposure (see Supplementary material A1).

Use of Food Consumption Survey

The third Dutch National Food Consumption Survey (DNFCS-3) provides a quantitative description of the daily food consumption over two consecutive days in 6250 individuals. Data were collected from April 1997 until April 1998 and were evenly spread over weeks of the year and days of the week (Kistemaker *et al.*, 1998). We used the list of nitrate-rich vegetables of the Dutch Food Centre (Voedingscentrum) in 2003 to identify vegetables rich in nitrate. Vegetables rich in nitrate that were reported in the DNFCS-3 to be eaten together with fish were celery, leek, endive, spinach, red beet, and Chinese cabbage. According to Westenbrink *et al.* (2005), nitrate concentrations in these vegetables varied from 300 mg/kg (cooked endive) to 1860 mg/kg (raw endive). Based on these concentrations, we calculated, for each individual, the associated intake of nitrate, and together with the type and amount of fish eaten, we estimated the amount of NDMA that would be formed after the reported meal, by applying the generic statistical relationship mentioned above. In these calculations, the nitrate exposure during a fish/vegetable meal in the DNFCS-3 (median: 0.32 mg/kg-bw/day) corresponded with the lower end of the nitrate exposure used in the *in vitro* gastrointestinal system to assess the formation of NDMA (0.1–0.5 mg/kg-bw/day). For further details, see Supplementary material A1.

Statistical Modeling of Exposure Data

The estimated amounts of NDMA (per kilogram body weight) for all participants of DNFCS-3 on both survey days were analyzed by the Statistical Exposure Model for Incidental Intakes (STEM.II; see Slob, 2006). In short, this model takes into account both the frequency of exposure days and the magnitude of the exposure on these days. Exposure frequency is defined as the fraction of days at which dietary exposure takes place, in this case, the frequency of fish + vegetable meals. The exposure frequency is assumed to vary among individuals according to a beta distribution, whereas the amounts of daily exposure are assumed to vary lognormally.

Distribution of Exposure Frequencies

The consumption data in the DNFCS-3 revealed that 102 out of the 12,500 persons in the survey had a meal combining fish with nitrate-rich vegetables on 1 of the 2 days and another 3 persons on both days. The associated NDMA exposure frequency was found to show a small but statistically significant trend with age, increasing from around 0.6 to 1.2% for the mean individual over the whole range of ages (Fig. 1). A beta distribution around the relationship with age was estimated to describe the variation in exposure frequency among individuals. One of the two beta parameters ("alfa") was assumed constant over age, the other ("beta") was estimated as a function of age, using the relationship: mean exposure frequency = alfa/(alfa + beta). In Figure 1, the trend with age in mean frequency (solid line) as well as the 5th and 95th percentiles (dashed lines) of the age-related beta distribution are shown. These percentiles represent the interindividual variability in exposure frequencies.

Distribution of Exposure Amounts

Figure 2 shows the estimated NDMA amounts per kilogram body weight, plotted against age. The higher intakes in children

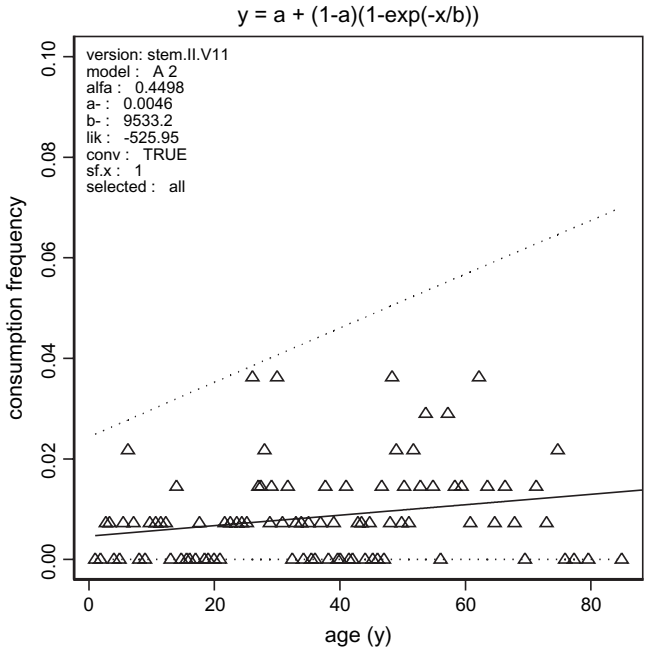

FIG. 1. Mean exposure frequencies of simultaneous consumption of fish and nitrate-rich vegetables (triangles) plotted against age. The increasing line represents the fitted regression function $y = a + (1 - a)(1 - \exp(-x/b))$. The dashed lines represent the 5th and 95th percentiles of the underlying beta distribution, representing variability in exposure frequencies among persons.

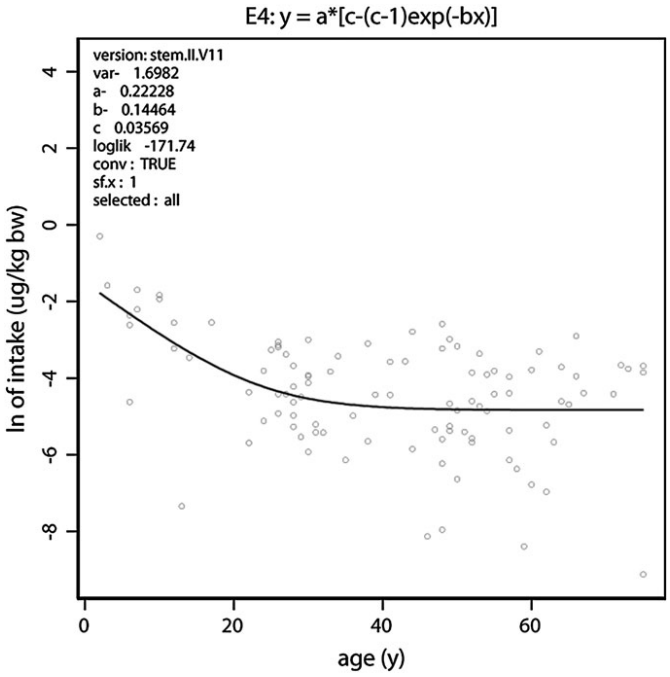

FIG. 2. Amounts of NDMA formed (μg/kg-bw) plotted on natural log-scale against age, for 105 DNFCS participants who consumed a meal with fish and nitrate-rich vegetables on at least 1 day, with a fitted regression function (with parameters *a*, *b*, and *c*). The within and between individual variance (for natural logs of NDMA amounts) was estimated at 0.15 and 0.06, respectively.

are in accordance with the usual pattern when intake is expressed per kilogram body weight.

Exposure Characterization

For chronic and acute risk assessment, the exposure needs to be characterized in different ways. For a chronic risk characterization, toxicity values should be compared with the long-term exposure within individuals. However, regarding NDMA formation from fish-vegetable meals, human individuals typically show many zero exposure days, interrupted with peak exposures on some of the days, and the question is how to translate these highly irregular exposures into a single measure of exposure, such that it can be compared with the constant doses applied in the chronic animal study. Current knowledge does not really answer this question, and therefore, we applied Haber's rule, i.e., the arithmetic average of both the nonzero and the zero exposure days during an individual's life is taken as the chronic measure of exposure. Although only two daily records are available per individual, the distribution of the long-term average intake can be estimated based on the (age-related) distributions for exposure frequency and exposure amounts, which were discussed above. The distribution of individual long-term exposures was estimated by sampling hypothetical individuals from both the distribution of exposure frequencies (Fig. 1) and that of exposure amounts (Fig. 2). For each sampled individual, the long-term exposure equals (according to Haber's rule) the exposure frequency times the exposure amount. Here, it was assumed that the amount of NDMA exposure does not depend on the frequency of exposure.

Figure 3 shows the 5th, the 50th, and the 95th percentiles of this long-term average exposure distribution as a function of age. The 95th percentiles decrease from 4.1 ng/kg-bw at age 1 year to 0.35–0.45 ng/kg-bw for adults > 30 years.

For an acute risk characterization, an estimate needs to be made of the fraction of days that the acute toxicity limit is exceeded. This fraction will depend on the individual and can be estimated from the same results (Figs. 1 and 2) by Monte Carlo sampling, see section "Risk Characterization: Acute Exposure."

DOSE-RESPONSE ASSESSMENT

Chronic Carcinogenicity Study

For NDMA, a very extensive chronic carcinogenicity study is available, with a total of 9120 animals distributed over 16 dose groups in both males and females (Peto *et al.*, 1991a,b). We used this study for dose-response analysis related to chronic exposure. Health-based limit values for NDMA-induced carcinogenicity have been derived in the past (e.g., 0.27–186 ng/kg-bw, RIVM, 1989; 0.02 ng/kg-bw/day, U.S. EPA, 1993). However, these limit values are not based on the more recent (high quality) carcinogenicity studies with

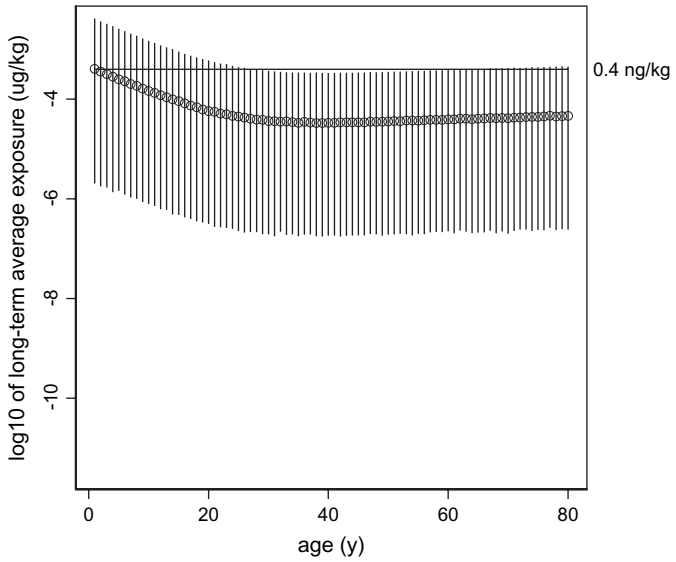

FIG. 3. Distribution of estimated long-term average NDMA exposure as a function of age. Dark marks: 50th percentiles. The vertical lines indicate the 5th to the 95th percentiles, reflecting the variability among individuals. The horizontal line indicates a chronic exposure limit of 0.4 ng/kg/day, derived from the data of Figure 4 using a conservative (low-dose linear) model (see text).

NDMA just mentioned. Therefore, we have ignored existing exposure limit values, and derived *ad hoc* limit values based on Peto *et al*. (1991a,b), for the purpose of this paper.

Dose-Response of Tumor Incidence after Chronic Exposure

The dose-incidence data from the chronic carcinogenicity studies were analyzed by the BMD approach. We used extra risk for quantifying the magnitude of the effect, which is defined as additional risk divided by the nonaffected fraction in the controls:

$$\text{Extra risk} = [P(d) - P(0)]/[1 - P(0)],$$

where P is the incidence probability and d any dose level. The BMD is the dose corresponding to a specified Benchmark Response (BMR), e.g., a 5 or 10% extra risk, calculated from the fitted dose-response relationship. The lower limit of the confidence interval around the BMD, i.e., the Benchmark Dose Lower bound (BMDL), represents the dose where the effect is smaller than the BMR with 95% confidence.

For fitting of dose-response relationships, the software of PROAST (Slob, 2002; www.proast.nl) was used. Next to the usual models (one-stage, two-stage, log-logistic, log-probit, Weibull, and Gamma), we applied so-called latent variable models (LVM). These models are based on an underlying continuous (latent) response variable, which in the case of cancer could be imagined to reflect the stage of the underlying (continuous) process of carcinogenesis. The point where visible tumors arise dichotomizes the underlying continuous response, resulting in a quantal response variable (yes/no tumor in each individual). Any continuous dose-response relationship may be used as reflecting the latent response variable in an LVM model. We used two nested families of continuous models, the exponential (E) and Hill (H) family of models (see Table 1), and determined the number of parameters to be included in the model by the principle of maximum likelihood ratio testing (see Slob, 2002).

The endpoint "total liver tumors" was found to be the most sensitive endpoint. Figure 4 shows these data, together with one of the fitted models (LVM-E4). Supplementary material A3 summarizes the BMDL10 values associated with the accepted models, showing that the variation among the BMDL10 values was small because of the good quality of the data. The lowest BMDL10 was 0.029 mg/kg-bw/day.

Dose-Response of Time to Tumor after Chronic Exposure

The study of Peto *et al*. (1991a,b) provides time-to-tumor data as a function of dose for various endpoints, including total liver tumors. Time-to-tumor data are continuous data, and we fitted the exponential as well as the Hill family of models (Table 1) to these data. From each of these families of models, a single model was selected based on the principle of likelihood ratio testing (Slob, 2002). Figure 5 shows the time-to-tumor data with

TABLE 1
The Exponential and the Hill Families of Models

Model	Number of model parameters	Model expression, response (y) as function of dose (x)	Constraints
Model 1	1	$y = a$	$a > 0$
Exponential family			
Model 2	2	$y = a \exp(bx)$	$a > 0$
Model 3	3	$y = a \exp(bx^d)$	$a > 0, d \geq 1$
Model 4	3	$y = a\,[c - (c - 1)\exp(-bx)]$	$a > 0, b \geq 0, c \geq 0$
Model 5	4	$y = a\,[c - (c - 1)\exp(-bx^d)]$	$a > 0, b \geq 0, c \geq 0, d \geq 1$
Hill family			
Model 2	2	$y = a\,[1 - x/(b + x)]$	$a > 0$
Model 3	3	$y = a\,[1 - x^d/(b^d + x^d)]$	$a > 0, d \geq 1$
Model 4	3	$y = a\,[1 + (c - 1)x/(b + x)]$	$a > 0, b \geq 0, c \geq 0$
Model 5	4	$y = a\,[1 + (c - 1)x^d/(b^d + x^d)]$	$a > 0, b \geq 0, c \geq 0, d \geq 1$

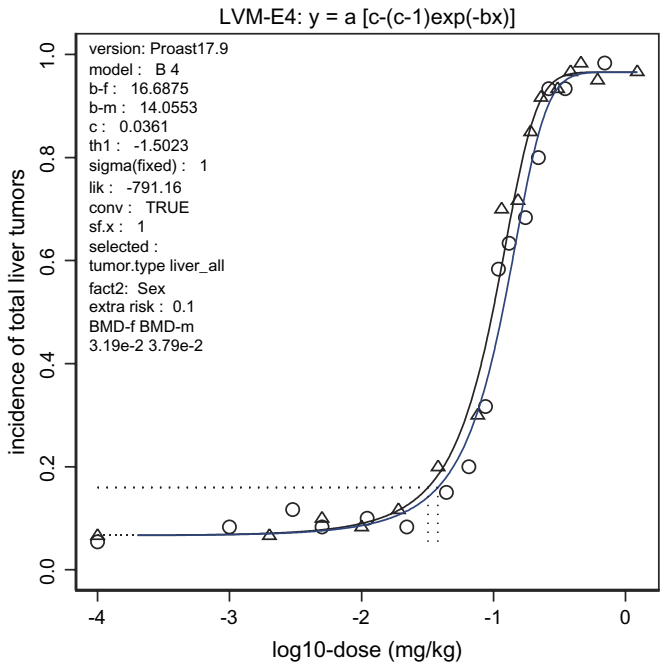

FIG. 4. Dose-response relationship for total liver tumors after lifelong exposure to NDMA via drinking water (milligram per kilogram per day). Data: Peto *et al.* (1991a). Δ = ♀; ○ = ♂. Lines: fitted LVM-E4 model for males and females, assuming parameters *a* and *c* identical but *b* different among sexes. The horizontal dotted line indicates the 10% extra risk level (compared with the estimated background response of 7.3%), the two vertical dotted lines the associated doses (BMDs) for females and males. Note that dose is plotted on log-scale, resulting in sublinear curves at the low range, which are however linear on the untransformed dose scale.

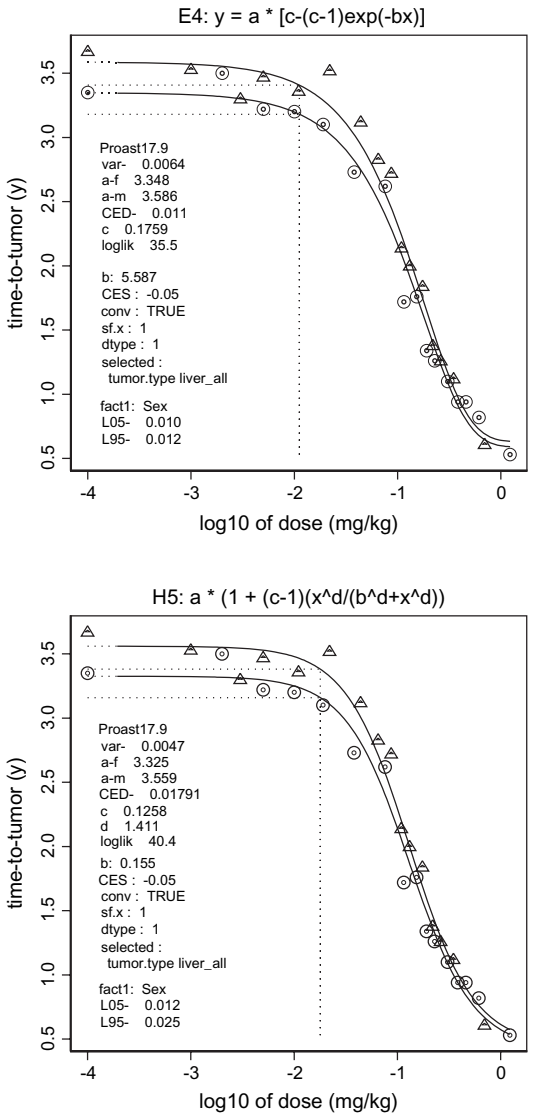

FIG. 5. Time to tumor as a function of dose, with exponential model E4 (upper panel) and Hill model H5 (lower panel) fitted to the data. Δ = ♂; ○ = ♀. Horizontal dotted lines indicate an effect size (BMR) of 5% decrease in time to tumor (one for each sex). Vertical dotted line: BMD05. Note that dose is plotted on log10-scale.

model E4 and model H5 fitted to them, respectively. For both models, a significant difference in background response between males and females was found, which in this case implies that "time to tumor" in the controls differed between both sexes. Furthermore, it was found that the dose-related change in time to tumor did not differ between both sexes, i.e., when normalized to the sex-specific background response, the two dose-response curves would coincide. As a consequence, the BMD does not depend on sex and can be estimated from the combined data for both sexes. The BMD analysis resulted in a BMDL05 of 0.010 mg/kg for the exponential and 0.012 mg/kg for the Hill model, where the BMR is defined as a 5% decrease in time to tumor, as compared with that in the controls.

The BMDLs associated with a 5% decrease in time to tumor were very similar between both fitted models, with a value of around 0.01 mg/kg. This small difference between the two fitted models reflects the good quality of the dose-response data.

It may be noted from Figure 5 that the time-to-tumor data at the lower doses are around 3.5 years, whereas the median lifespan in this study was observed to be 2.5 years in females and 2.7 years in males (Peto *et al.*, 1991b). The reason is that Peto *et al.* (1991a) estimated the median time to tumor based on fitting Weibull "survival" curves to the time-to-tumor data for each dose group, thereby treating the animals that died without liver tumors as censored data. For instance, an animal that dies at the age of 2 years without liver tumors is included in the statistical analysis as the observation: time to tumor > 2 years. In this way, it is assumed that tumor-free animals died because of some competing cause, whereas in a world where such competing causes were nonexistent, every animal would at some point in time get a liver tumor. This assumption is supported by the fact that the time-to-tumor data when plotted as survival curves were all parallel (on log-age scale) among the 16 dose groups and could be well described by a single Weibull survival function that only differed in location (i.e., median age). In other words, the median ages reported by Peto

et al. (1991a) are, for the lower doses, virtual values that cannot be reached in reality because of competing causes of death.

Dose-Response of Tumor Incidence after Acute Exposure

For acute exposure, we used the study by Driver *et al.* (1987). In this study, a single dose of NMDA was administered to weanling rats, and 20–24 months later, the animals were examined for tumors. The dose-incidence data reported for the endpoint kidney tumors were subjected to a BMD analysis (see Supplementary material A3). Figure 6 shows these data, together with one of the fitted models (Weibull). The variation among the BMDL10 values related to the various accepted models was small, the lowest being 11 mg/kg.

RISK CHARACTERIZATION: CHRONIC EXPOSURE

We now discuss various approaches of risk characterization after chronic exposure of endogenously formed NDMA.

Margin of Exposure Approach

The margin of exposure (MOE) approach measures the distance (ratio) between human exposure and some toxicity measure, denoted as the PoD (point of departure) or RP (Reference Point). See Barlow *et al.* (2006) or O'Brien *et al.* (2006) for further discussions of this approach. The current view is that the BMDL10 is the preferable PoD (Benford *et al.*, 2010; ILSI, 2009). Using the BMDL10 for (total liver) tumor incidence (0.029 mg/kg-bw), and the 95th percentile of the long-term exposure distribution (4.1 and 0.40 ng/kg-bw for children of 1 year of age and adults, respectively; see Fig. 3), results in MOEs of 7000 for children 1 year of age and 72,500 for adults. For a discussion of how to deal with the MOEs in children or adults, see "Discussion of the Hazard Characterization."

Fraction of the Population Exceeding de minimis *Risk Level*

For all models that resulted in an acceptable fit the dose (BMDL) associated with a 10^{-6}, extra risk was derived. The resulting BMDLs for 10^{-6} risk varied 1400 ng/kg-bw for the log-probit model and 0.4 ng/kg-bw for the LVM-E4. Table 2 provides BMDLs associated with other risk levels for these two models, which show that the model LVM-E4 has a linear behavior at low doses, whereas the log-probit model is sub-linear. Because both models adequately describe the available dose-response data, this illustrates that even for this excellent data, it cannot be decided whether the dose-response at the lower end is in fact linear or not. But, by assuming that a linear dose-response would be worst-case (O'Brien *et al.*, 2006), the value of 0.39 ng/kg-bw could be regarded as a lower bound estimate of the dose associated with a 10^{-6} risk level. In the discussion below, this value is rounded off at 0.4 ng/kg-bw.

The conservative estimate of 0.4 ng/kg-bw for a *de minimis* risk can be compared with the distribution of long-term exposures in the population. As Figure 3 shows, about 50% of the very young children have an estimated long-term average exposure above the level of 0.4 ng/kg-bw. For adults (> 25 years of age), this fraction is estimated to be about 5%. For a discussion of how to deal with these results for children or adults, see "Discussion of the Hazard Characterization" section.

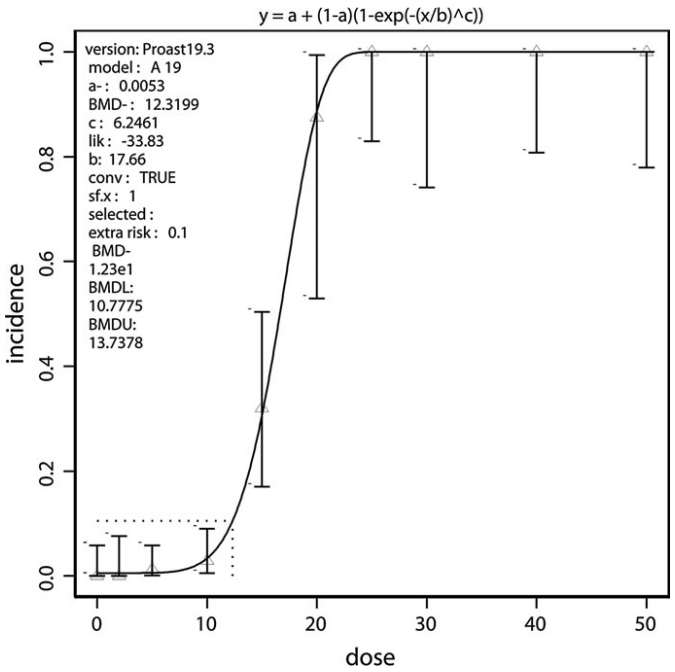

FIG. 6. Dose-response relationship between single administration of NDMA (milligram per kilogram body weight) and the induction of mesenchymal kidney tumors in the rat 20–24 months after the administration. Data from Driver *et al.* (1987). Curve: fitted Weibull model. Dotted lines indicate the BMD_{10} and the BMR of 10% extra risk. Vertical lines: two-sided 90% confidence intervals for the observed incidences.

Distribution of Cancer Risks in the Human Population

As another approach of characterizing the risk, we estimated the overall risk distribution by calculating the cancer risk at each individual Monte Carlo exposure result underlying the exposure distribution shown in Figure 3. The individual cancer

TABLE 2
BMDLs for Various Risks Derived with Log-Probit Model and LVM-E4. The Underlying Dose-Response Data are Shown in Figure 4

Extra risk	Log-probit model BMDL (mg/kg)	LVM-E4 BMDL (mg/kg)
0.10	0.029	0.031
0.05	0.021	0.016
0.01	0.012	0.38×10^{-2}
0.005	0.0093	0.19×10^{-2}
10^{-3}	0.0034	0.39×10^{-3}
10^{-6}	0.0014	0.39×10^{-6}

risks were calculated by applying the LVM-E4 as it represents the worst-case dose-response at low doses. From the resulting risk distributions, two are shown in Figure 7: for the age categories of 5 and 30 years. They reveal that the cancer risk will vary considerably among individuals, but the risks would rarely exceed 10^{-5} in the highest exposed individuals.

Characterizing Human Risk in Terms of Time to Tumor

Although cancer risks are usually expressed in terms of a potential increase in the incidence of cancer, the human health impact from exposure to carcinogens could also be expressed in terms of a potential decrease in the time that is needed to develop the tumor, i.e., the time to tumor. The study of Peto *et al.* (1991a,b) offers the opportunity to explore this idea, by using the dose-response of time to tumor in rats (see Fig. 5).

For the lower bound estimate of the dose level associated with a 10^{-6} risk, i.e., 0.4 ng/kg-bw, we can estimate the percent decrease in time to tumor in the same population of experimental animals at that particular dose level. For the exponential model E4, the percent decrease in time to tumor is estimated at 0.00021% and for the Hill model H5 at 1.7E-06% (see table 3). In other words, given a median time to tumor of 3.5 years for control rats in this study, the decrease in time to tumor at the dose associated with a 10^{-6} risk would be around 3.8 min according to model E4 and 0.032 min according to model H5. Assuming that humans show a similar dose-response for both tumor incidence and for time to tumor as observed in rats, the decrease in time to tumor in humans at the 10^{-6} risk level would be 2.2 h according to model E4 and 1.1 min according to model H5. Here, we assumed a factor of 35 for scaling a rat lifespan to the human lifespan.

To further explore the relationship between health impact expressed in terms of cancer incidence versus in terms of time to tumor, similar calculations were done for a range of nominal risk levels (10^{-1} to 10^{-6}). For each of these risk levels, the associated doses (BMDs) were calculated according to the most conservative model (LVM-E4 model) fitted to the tumor incidence data. Next, for each of the resulting doses, the associated percent decrease in time to tumor was calculated from the time-to-tumor dose-response. The results are summarized in Table 3, for both fitted time-to-tumor models. Note that in this table, not the lower confidence bounds (BMDLs) but the best estimates (BMDs) were calculated. The table shows that the combination of the most conservative model fitted to the incidences, and the most conservative model fitted to the time-to-tumor responses results in low estimates for the decrease in time to tumor at *de minimis* risk levels. Even for a risk level of 10^{-4}, the conservative estimate of the associated reduction in time to tumor in humans amounts to around 9 days.

RISK CHARACTERIZATION: ACUTE EXPOSURE

For acute exposures, we applied the following approaches of risk characterization.

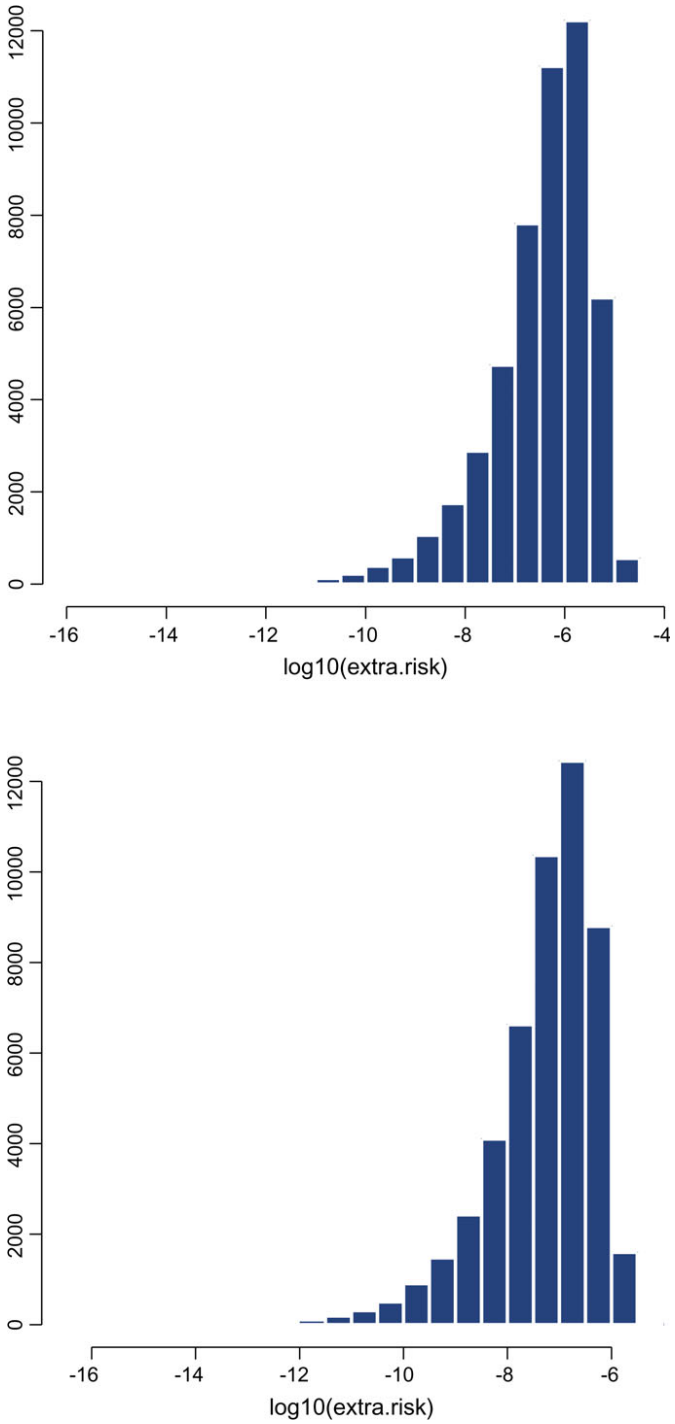

FIG. 7. Distribution of cancer risks in the human population for age = 5 years (upper panel) and 30 years (lower panel), using the most conservative dose-response model (LVM-E4) that was fitted. Number of Monte Carlo drawings: 50,000. 95th percentile in upper plot is 6×10^{-6} and in the lower plot 8.3×10^{-7}.

MOE Approach

Given the incidental high-peak exposure to endogenous NDMA formation in the human population, a risk characterization based on the results from the acute carcinogenicity

TABLE 3

Relationship between Various Risk Levels and the Associated Reduction in Time to Tumor, Expressed as a Percent Decrease Compared with the Controls, and Expressed in Minutes for Rats and In Days for Humans, Deduced from the Dose-Responses for Tumor Incidence and for Time to Tumor for NDMA

	Reduction in time to tumor					
	Model E4			Model H5		
Extra risk	Rats (% change)	Rats (min)	Humans (days)	Rats (% change)	Rats (min)	Humans (days)
10^{-1}	13	247,496	6016	11	193,721	4708
10^{-2}	1.9	35,802	870	0.70	12,838	312
10^{-3}	2.1E-01	3784	92	2.9E-02	535	13
10^{-4}	2.1E-02	381	9	1.1E-03	21	0.51
10^{-5}	2.1E-03	38	0.9	4.4E-05	0.81	0.020
10^{-6}	2.1E-04	3.8	0.09	1.7E-06	0.032	0.0008

study by Driver *et al.* (1987) is relevant. As discussed above, the BMDL10 resulting from this study was 11 mg/kg-bw. The exposure analysis showed that most of the estimated amounts of NDMA in the participants of the food survey were below 0.10 μg/kg (see Fig. 2). Hence, the MOE for acute NDMA exposure after a fish + vegetable meal would be greater than 100,000.

Fraction of the Population Exceeding de minimis *Risk Level*

Linear extrapolation from the BMDL10 to a 10^{-6} risk level would lead to an acute exposure limit of 110 ng/kg-bw. Figure 8 shows the fraction of individuals as a function of the associated fraction of days at which this limit value is exceeded by that fraction of individuals, for the subgroup of 5-year-old children. It can be read from the plot that up to 1% of this age class would exceed the limit value by at least 2% of the days. In adults, the percentage of days at which this limit value is exceeded by the upper 99th percentile of the population was close to zero (data not shown).

The accepted dose-response models were also used to estimate the doses associated with 10^{-6} extra risk. They ranged between 6.1 μg/kg and 4.3 mg/kg. This model uncertainty is comparable with that found for the chronic dose-incidence data. However, a difference with the analysis of the chronic dose-response data is that none of the models showed linear behavior at low doses. This indicates that the assumption underlying a linear low-dose extrapolation most likely is unrealistic for acute exposure to NDMA. Therefore, taking linearity as a worst-case situation would be demonstrably unrealistic in this case. Estimating low-risk doses from the various models with acceptable fits leads to largely different estimates because of the associated extrapolation over various orders of magnitude. Hence, we considered the option of quantifying cancer risks in the human population because of acute NDMA exposure not appropriate here.

DISCUSSION OF THE EXPOSURE ASSESSMENT

The exposure assessment for endogenous NDMA as presented in this paper is, among others, i.e., food consumption data, characterized by some specific uncertainties because of the *in vitro* formation data. The validation of the *in vitro* formation of endogenous NDMA with human experimental data is difficult, not only for ethical reasons but also because of the rapid absorption of NDMA from the stomach once it has been formed. Fortunately, the *in vitro* gastrointestinal system appears to closely simulate the human physiological conditions after eating a meal, by mimicking physiological parameters such as the pH, body temperature, peristaltic movements, and secretion of digestive enzymes in a controlled and standardized way (Krul *et al.*, 2004). Furthermore, previous experiments showed that the absorption and distribution of food mutagens (heterocyclic aromatic amines) in the *in vitro* system were comparable with the results of *in vivo* studies in mice and humans (Krul *et al.*, 2000).

In Vitro Experiments

The formation of NDMA as measured in the *in vitro* model with cod was found to be rather variable, up to a factor of 5. This might be the result of using different codfish batches. This variation was not taken into account in the exposure assessment. For chronic exposure assessment, where a long-term average exposure is relevant, this variation would largely cancel out. But also for acute exposure assessment, we found that temporal fluctuations in NDMA amounts formed in single meals did not have much impact on the results. The reason is that the fraction of days where the exposure limit is exceeded is almost completely driven by the exposure frequency distribution: given a fish + vegetable meal, the probability is large that the exposure limit will be exceeded on that day. In other words, the amount of NDMA formed because of a fish + vegetable

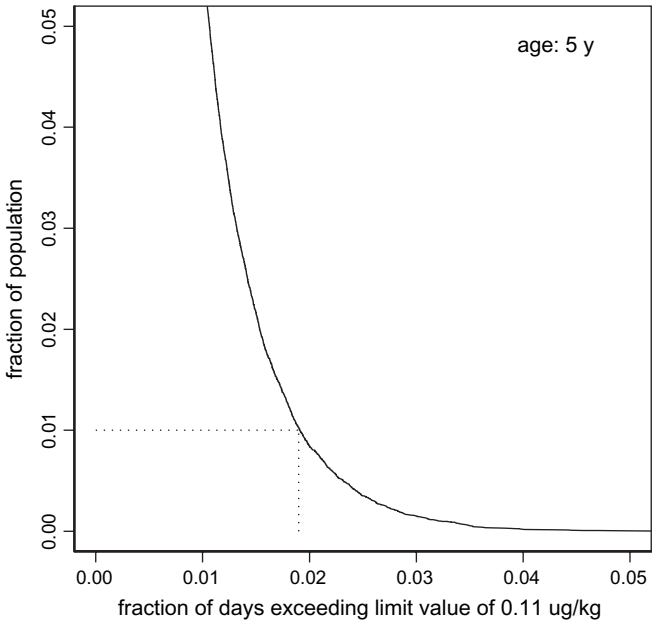

FIG. 8. The fraction of the subpopulation (y-axis) in 5-year-old children plotted against the fraction of days (x-axis) at which the acute exposure limit of 110 ng/kg-bw is exceeded. This curve should be read analogous to a survival curve, e.g., less than 1% of the population exceeds the limit value more than around 2% of the days. Calculations are based on the results shown in Figures 1 and 2, using the methodology of Slob (2006).

meal is generally so high that it amply exceeds the exposure limit. Hence, the acute exposure assessment is rather insensitive to moderate variations in NDMA formation.

The smallest amount of cod investigated in the *in vitro* model was 30 g. It might be argued that there is a fairly high uncertainty regarding the shape of the fitted regression function at amounts of cod lower than 30 grams (see Supplementary material A1). As the cod data are used as the basis for calculations of all other fish species, the same might hold for the other species. This might introduce considerable uncertainty in estimated NDMA formation associated with the consumed amounts of fish as reported in the food survey. However, in the DNFCS-3, the reported amounts of fish were lower than 30 grams in only 10 out of the 105 cases where fish was consumed together with nitrate-rich vegetables. So, the uncertainty in NDMA formation at relatively low amounts of fish consumed may not substantially disturb the outcomes of the overall risk assessment.

Similarly, NDMA formation was studied at salivary nitrite flows corresponding with an *in vivo* nitrate exposure lower than the ADI in only 8 of the 55 experimental runs with the *in vitro* system. In the DNFCS-3, consumed nitrate amounts from nitrate-rich vegetables (consumed together with fish) are mostly below the ADI (45% of the ADI, on average; the ADI for nitrate was exceeded in only 10 of the 105 cases). However, the *in vitro* results showed a linear relationship of NDMA formation over a wide range of nitrate exposures (for details see equation for NDMA formation in Supplementary material A1).

Food Consumption Data

The food consumption survey (DNFCS-3) showed only 105 out of 12,500 person-days where a combination of fish and nitrate-rich vegetables in the same meal was consumed. It might be that this information is insufficient to reliably estimate the exposure frequency distribution in the population. Using simulations, Slob (2006) showed that an exposure frequency distribution with an expected frequency as low as 0.0065 can still be estimated reasonably well from food surveys like the DNFCS-3. For fish + vegetable meals, we found a mean frequency of 0.01–0.03 (see Fig. 1).

From the 105 person-days with a fish + vegetable meal was consumed, only 3 individuals did so on both days. Hence, the estimate of the variance within persons (between days) is very poor in this case.

Endogenous NDMA Formation and Matrix Effects

NDMA formation may be modified by the addition of food components (Krul, 2001; Krul and Havenaar, 2005; Krul *et al.*, 2004). Indeed, a whole food matrix clearly reduces gastric NDMA formation, but it does not fully neutralize it. Single food items for which a reduction in NDMA formation was found are orange juice, black tea, and ascorbic acid (Krul *et al.*, 2004; Vermeer *et al.*, 1998). Inhibitory effects were taken into account in the present risk assessment, but as the corrections applied are based on limited data, they may involve quite some uncertainty.

DISCUSSION OF THE DOSE-RESPONSE ASSESSMENT

The available dose-response data from the chronic carcinogenicity study were of exceptional quality. As a result, the BMDL10 values associated with the various accepted models were very close to each other. Nonetheless, even for such excellent dose-response data, the problem of low-dose extrapolation persists: The dose associated with a 10^{-6} extra risk varied nearly four orders of magnitude among the accepted models. In addition, despite the large number of doses (16), nothing could be said about the dose-response at low risks, not even if it would be linear or sublinear: Both options were in accordance with the dose-response data, i.e., the data did not allow a discrimination between linearity or nonlinearity.

For the acute dose-incidence data, the shape of the observed dose-response was such that a linear relationship, e.g., from the BMD10 downwards, could be rejected. This is because of the extreme nonlinearity of the dose-response at observable incidences. However, the assessment that the dose-response is in fact sublinear is not very helpful in a quantitative sense: the extent to which it is sublinear, and hence, the quantitative dose-response below the observable incidences remains uncertain.

The most innovative aspect of the available dose-response information for NDMA is the time-to-tumor data. Such data, by

being continuous, not only represent better information than cancer prevalence data from a statistical point of view, they also offer the opportunity to represent cancer risks in terms of time to tumor (see "Discussion of the Hazard Characterization" below). However, time to tumor data do involve a specific analytic difficulty: At lower doses, many of the animals die for reasons other than cancer and do not reach the stage of visible tumors. Statistically, this can be solved by the concept of censored data: An animal that dies tumor free at age 2 years is considered as the observation that the time to tumor in that animal is larger than 2 years. This implies the assumption that all animals would get the tumor at some point, if only they were able to live long enough. The Peto *et al.* (1991a,b) study was continued until all animals had died naturally or had been killed for humane reasons. In current regulatory practice, carcinogenicity studies are usually interrupted after 2 years, which increases the number of censored data. But even in these studies, a dose-response analysis as performed in the present paper may be feasible. The loss of information related to the censoring of time-to-tumor information will be captured in the outcomes from the statistical results, resulting in wider confidence intervals for the BMD. It does not represent a fundamental difficulty, however.

DISCUSSION OF THE HAZARD CHARACTERIZATION

Chronic Exposures

The major difficulty in cancer risk assessment is the fact that acceptable risk levels are various orders of magnitude lower than can be measured in animal studies. For the specific case of NDMA, the available carcinogenicity studies are extremely good. Therefore, we took the opportunity to use these data with the purpose of comparing different risk characterization approaches and of exploring the possibilities and limitations of cancer risk assessment for both NDMA itself and in general.

The MOE approach has been recently introduced as a general approach of risk characterization of genotoxic carcinogens (Barlow *et al.*, 2006; Benford *et al.*, in prep; O'Brien *et al.*, 2006). For chronic exposure, we assessed the MOE for adults at 72,500 and for children at 7000. The MOE approach may be considered as a useful method of ranking and prioritization, but it is difficult to interpret an MOE in terms of health risks (Barlow *et al.*, 2006).

The fact that the MOE in children is lower than that in adults should not be interpreted as an indication that children are at higher risk than adults. Any such conclusion would be inadequate as the underlying rat study tries to estimate risks after lifelong exposure. Therefore, whatever the method of risk characterization, the answer always refers to individuals who have been exposed during their whole life. It might be that exposures early in life are in fact driving the cancer risk later in life; in that case, the MOE assessed in children would be more relevant. Or it might be that exposures later in life drive the cancer risk and then the MOE in adults would be more relevant. In reality, both the exposure early and later in life may be relevant and then the "real" MOE should be somewhere in between. Therefore, the MOEs for children and adults should be interpreted as margins of uncertainty in the single MOE, which relates to people after chronic exposure, without distinction between age groups.

An additional point to be made here is that the doses in the underlying rat study were higher in young rats as well, when expressed in milligram per kilogram body weight (concentrations in drinking water were kept constant during the study while young rats were drinking more water). Therefore, it could be argued that the higher exposure in children had also occurred in the young rats, so that the age-related change in exposure is in fact already accounted for in the animal study. However, in humans, the higher exposure in children amounts to a factor of around 10, whereas in rats, the difference was no more than a factor of 1.5. Therefore, the exposure in humans integrated over the whole lifespan will be higher than that in the rats, in any given situation where the exposure in adult people is comparable with that in adult rats.

The dose-response data, with 16 different dose levels, provide excellent information on the dose-response, but nonetheless, they did not give any clue if the dose-response was linear or nonlinear at low doses, i.e., below the range of observation. Based on general biological arguments (O'Brien *et al.*, 2006), it seems not unreasonable to regard a linear dose-response at the lower end as a worst-case situation for tumor incidences. Therefore, we derived the dose associated with a 10^{-6} risk by linear model extrapolation and regarded this as a lower bound estimate of that dose. In this way, it should be possible to at least produce worst-case estimates of cancer risks in humans, i.e., they should be considered as conservative. We found that one of the fitted models (LVM-E4) showed linear behavior at doses below the range of observation. Therefore, it cannot be excluded that the dose-response is actually close to linear between the background response and 10% extra risk (= the PoD for linear extrapolation), so that the associated estimated dose at 10^{-6} risk could be close to reality.

Comparison of this 10^{-6} exposure limit to the results from the exposure assessment showed that 5% of the adults exceeded this limit value, whereas 50% of the children did so (see Fig. 3). As just discussed for the MOE approach, these results for children and adults should be interpreted as margins of uncertainty in the (single) MOE because of the fact that exposure is a function of age, which as a whole contributes to the cancer risk (but we do not know how).

A next question raised by these results is what might be the risks for those individuals that exceed the exposure limit? This question was addressed by calculating the distribution of risks (Fig. 7) associated with the variability in human exposures. The upper 95th percentile of the population (regarding endogenous NDMA exposure) was estimated at around 8×10^{-7} for adults and 6×10^{-6} for children. So, given the uncertainty how exposures at different ages contribute to the cancer risk, we could state that the estimates of risk are somewhere between

8×10^{-7} and 6×10^{-6} for the 95th percentile of the human population.

In an attempt to get more insight in the relevance of such risk levels in terms of life expectancy, we analyzed the time-to-tumor data provided in the study by Peto *et al.* (1991a,b). This analysis provided a useful result: the relationship between cancer risk and decrease in time to tumor at the same dose. As Table 3 showed, a 10^{-6} risk would be associated with small changes in time to tumor. The most conservative (and linear at low doses) model fitted to the time-to-tumor data predicted a reduction in time to tumor by around 4 min in rats. Furthermore, even a risk of 10^{-4} would imply a reduction in time to tumor of around 6 h in rats. For a human lifespan, this would be analogous to around 9 days. So, even if the dose-responses for both tumor incidence and time to tumor are in reality linear at lower doses, and even if the high but relatively short exposures in children are decisive for final cancer risks, then the estimated loss of healthy lifetime would still be less than 9 days in a large fraction of the population.

Acute Exposures

We found that in young children, a small fraction of the population exceeded the acute exposure limit of 110 ng/kg, e.g., 1% of children 5 years of age would exceed this limit at around 2% of the consumption days. This result is not easy to interpret. On the one hand, 2% of the days may be considered as a high percentage, given the fact that the exposure limit was based on a study where the juvenile animals received only one single dose. On the other hand, the limit of 110 ng/kg (associated with 10^{-6} risk) was estimated by linear extrapolation, whereas the dose-response data for the acute study indicate that this procedure almost certainly overestimates risks at low doses. Therefore, this estimated fraction of days must be regarded as an overestimate. In addition, when it is assumed that the relationship between time-incidence and time to tumor in the acute exposure situation is comparable with the chronic exposure situation, risk levels of 10^{-6} would hardly decrease the time to tumor in humans, and an exposure limit based on such low risks might be overly protective.

From Rats to Sensitive Humans

Finally, it should be noted that so far, potential inter- and intraspecies differences have not been taken into account. There is growing awareness that such differences should be taken into account for cancer risk assessment, just like in noncancer risk assessments (e.g., Barlow *et al.*, 2006). In the case of NDMA, mode of action (MOA) considerations might be helpful in evaluating the relevance of the dose-response for carcinogenicity in rats for humans. For instance, the repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) appears to be a key factor in carcinogenicity of methylating agents like NDMA (Allay *et al.*, 1999; Bugni *et al.*, 2009; Iwakuma *et al.*, 1997; Pegg, 2000; Sekiguchi *et al.*, 1996). With regard to the liver, the rat possesses basal and inducible MGMT, with inducibility starting from NDMA doses of 0.7 mg/kg-bw/day. In contrast, mice only possess basal MGMT activity. Not surprisingly, O^6-methylguanine (O^6-MeG) accumulates in mouse liver DNA as doses exceeding 0.7 mg/kg-bw/day, whereas in rats, such an increase is not observed (Lindamood *et al.*, 1984). However, as the maximum dose tested in the Peto just lies at the level where inducible MGMT activity starts to occur, the chronic carcinogenic response used in this paper, i.e., the Peto study, has not been influenced by this mechanism, i.e., this response being determined by the administered dose at constant basal MGMT activity. As it has been found that the repair efficiency of rat and human MGMT is comparable (Liem *et al.*, 1994), the rat liver carcinogenicity data may be considered an appropriate model for humans, thereby favoring a relatively low assessment factor for interspecies differences in carcinogenicity. Potential differences in MGMT levels among people appear to be unknown, so that a chemical-specific intraspecies adjustment factor seems hard to establish. In this context, Supplementary material A5 provides some more information on the MOA of NDMA.

In general, when chemical-specific information is lacking, a simple approach would be to apply the usual assessment factors of 10, but this would add to the already conservative nature of cancer risk assessment. Therefore, the next step would be to take inter- and intraspecies differences into account in a probabilistic way (e.g., Slob and Pieters, 1998). Such an approach would nicely link to the probabilistic results as presented in this study. In addition, some of the conservative assumptions in the risk assessment presented here may be included in a probabilistic way.

CONCLUSIONS

Though it may be argued that considerable uncertainty remains in the calculation of the endogenous NDMA formation and that the *in vitro* formation of NDMA in the human stomach out of nitrite and fish constituents can by no means be validated in *in vivo* studies, the results of the present study appears to give the scientifically best possible approach of evaluating the potential cancer risk from a fish/nitrate-rich vegetable meal. Apart from the specific uncertainties in the exposure assessment, a risk assessment of endogenous NDMA is faced with the usual uncertainties in cancer hazard characterization, even though the carcinogenicity studies available for NDMA are of exceptional quality. We have used the latter fact to elaborate further on the possibilities and limitations of current risk assessment approaches, and, by taking the conservative nature of the approaches into account, we conclude that the consumption of fish/vegetable meals appears to lead to only marginal increases of human cancer risk.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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